EXAMINING SUBMERGED AND SOLID-STATE CULTIVATION COURSE OF HYDROLYTIC ENZYMES PRODUCTION FROM WHEAT CHAFF

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Abstract
Agricultural waste represents an interesting raw material for biotechnological processes nowadays, due to its low price, favorable composition and availability. Wheat chaff, as a lignocellulosic by-product of wheat processing could be a suitable renewable source for producing hydrolytic enzymes for second generation ethanol production technologies. The aim of this work was to investigate the course of *Trichoderma reesei* cultivation for hydrolytic enzymes production by submerged and solid-state techniques on medium based on wheat chaff. Results show that the monitored values (hydrolytic enzymes activities, total protein content and reducing sugars content) vary significantly with cultivation time, thus there is a need for further optimization of this process parameter.

Introduction
With its composition, wheat chaff is a very attractive raw material for the production of enzymes [1]. On the other hand, the previous use of this by-product of grain processing was only as fodder for cattle. Therefore, the question arises of the possibility of obtaining greater economic and environmental benefits by using a given raw material for the production of a high value product, such as enzymes, with the valorization of other process outputs to achieve the concept of cleaner production, or zero emission concept. It is the simulation models of production plants that represent an auxiliary tool for performing economic analysis and other calculations related to bioprocess, which are important for their design [2]. For the purposes of generating such models, and later the control of the bioprocess itself, it is necessary to know the kinetics and kinetic parameters related to a given process. Determining these kinetic equations only makes sense when the bioprocess is performed under optimal conditions [3]. In order to optimize the process, it is necessary to study in detail the production of enzymes by cultivating fungi on by-products of grain processing at different process parameters [4].

The aim of the research completed within this work is to investigate the submerged and solid-state cultivation course for producing hydrolytic enzymes by cultivating fungi on media that contain wheat chaff as a basis for nutrient media. The results obtained will provide data on which process parameters will need to be further optimized.

Experimental
Trichoderma reesei QM 9414, which are kept in the collection of cultures at the Faculty of Technology Novi Sad, was used as a producing strain. Refreshing of the fungi was carried out on the potatoes dextrose agar (PDA) by incubating them for 3-4 days at 28 °C. The inoculation of the nutrient media was carried out with a pre-prepared spore suspension in a sterile saline solution containing 10^6 spores/g. For the purpose of experiments, 10 % of the inoculum was added to the liquid substrates, and for the solid-state substrates, the same volume of the spore suspension was sprayed over their surface.

For the purpose of research, the by-product of wheat processing (wheat chaff) was used to prepare nutrient media. The raw material was obtained from the local wheat processing plant (mill) "Žitopromet - Mlin" a.d. Senta.

The composition of liquid substrates for the submerged cultivation technique (SmF) on wheat chaff with the aim of selecting the producing strain was 3 g of wheat chaff, 0.5 % $(NH_4)_2SO_4$ and 1.36 % K_2HPO_4 in 100 mL of distilled water.

For cultivation on solid substrates (SSF) with the aim of selecting the producing strain, the same amount of raw material (3 g) was suspended in the same amount (100 mL) of a water solution containing 0.5 % $(NH_4)_2SO_4$ and 1.36 % K_2HPO_4 like for the liquid media. After 15 minutes of mixing, the pH value was checked and corrected to 4.5 ± 0.1 by adding 1 % NaOH or 1 % H_2SO_4. After an additional 15 min of stirring, the suspension was allowed to stand still so that the solid phase could settle in the gravitational field. The liquid phase was decanted and the residue used as a solid substrate for the production of enzymes. In this way enzymes have been produced from the same amount of raw material used, i.e. 3 g of wheat chaff, as well as the same preparation method (100 mL of prepared salt solution), so that the obtained results could be comparable [5].

Sterilization of the prepared media was carried out in an autoclave at a temperature of 121 °C and a pressure of 2.1 bar for 20 min.

Production of enzymes by fungi cultivation for both submerged and solid-state techniques was carried out in 300 ml Erlenmeyer flasks for 7 days at a temperature of 28 ± 1 °C.

After cultivation on solid media, the products of strain metabolism were extracted with 100 mL 0.1 M acetate buffer (pH 5.0) with constant mixing at 200 rpm during 30 min at a constant temperature, in order to equal the liquid volume with the submerged cultivation broths. Separation of solid and liquid phase after extraction of solid media as well as the submerged cultures was carried out by filtrating through a qualitative filter paper. Obtained filtrates were subjected to the standard analysis of cultivation media.

The intensity of hydrolytic action of the cultivation liquids and solid extracts towards cellulose and xylan were assayed separately for each substrate by measuring the release of reducing sugars using the DNS (3,5-dinitrosalicylic acid) method [2].

Results and discussion

In addition to the appropriate nutrient medium and production microorganism, the success of fermentation also depends on the knowledge of the values of the desired parameters in certain phases of the process, i.e. the knowledge of the cultivation course [6]. Knowing the course of a bioprocess helps in its better understanding, facilitates its translation on a larger scale (scale-up) to the final industrialization, and thus its control and management. For this reason, experiments were performed using submerged and solid-state cultivation of *Trichoderma reesei* on a substrate based on wheat chaff, with the aim of analyzing the activity of hydrolytic enzymes (amylase, cellulase and xylanase), total protein content and reducing sugars at defined time intervals of bioprocesses. Figures 1 and 2 show the results of cultivation course tests for submerged and solid-state techniques, respectively.

Based on the results shown in Figure 1, it can be seen that the activities of the tested hydrolytic enzymes in the culture medium have an intense increase in the first 48 h, followed by a slight

increase or stagnation until the end of cultivation. According to the same principle of the sigmoidal curve, which is characteristic for enzyme systems [7], the total protein content also changes, with the moment of transition from sudden to weaker intensity of increase of this value a little earlier in relation to the activities of tested enzymes, i.e. about 32 h. The content of reducing sugars in the first four hours has an intensive growth, when it reaches its maximum value of 12.4895 mg/mL, then sharply decreases to about 2 mg/mL at 24 h and by the end of cultivation this value does not change drastically.

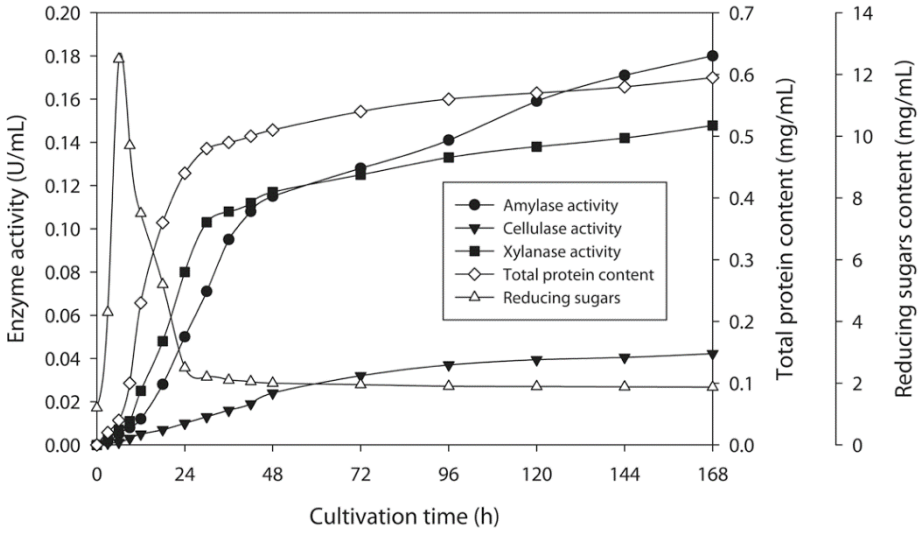

Figure 1. Enzyme activity (amylase, cellulase and xylanase), total protein and reducing sugars content as functions of submerged cultivation time of *Trichoderma reesei* on a nutrient medium with wheat chaff

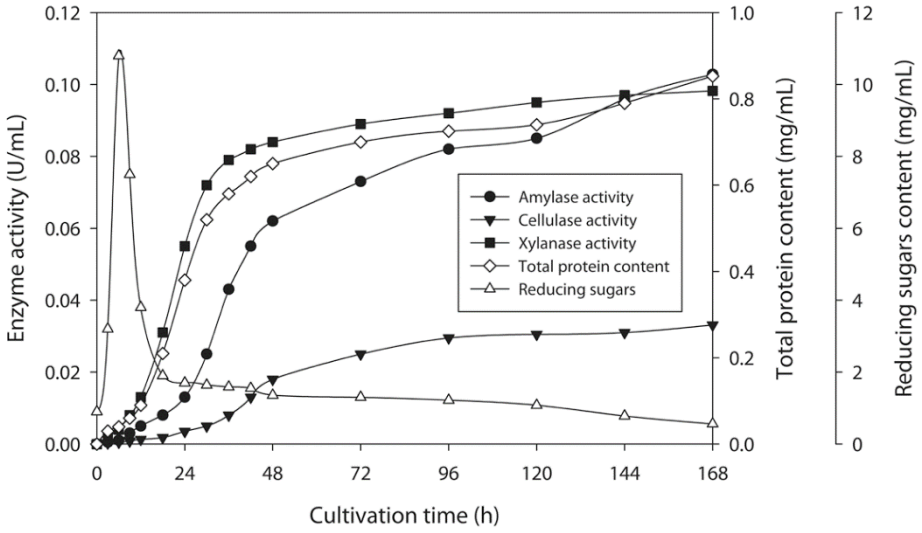

Figure 2. Enzyme activity (amylase, cellulase and xylanase), total protein and reducing sugars content as functions of solid-state cultivation time of *Trichoderma reesei* on a nutrient medium with wheat chaff

Analysis of amylase, cellulase and xylanase activity in samples taken during fungi cultivation on a solid nutrient medium based on wheat chaff (Figure 2) shows that the values first increase intensively up to 48 h, then increase slightly until the end of cultivation. As with submerged cultivation, the total protein content first has a sharp increase, but this time up to 48 h, and then

a slight increase up to 168 h. The maximum value of the reducing sugar content was reached already in 4 h of cultivation, after which it falls to a value below 2 mg/mL already in 18 h of cultivation and continues to fall to a value of 0.5 mg/mL until the end.

Conclusion

Examining the course of two cultivation techniques for producing hydrolytic enzymes by fungi on a nutrient medium based on wheat chaff, it was pointed out that there is a possibility of shortening the cultivation time, i.e. the need for further optimization in the form of process parameters.

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